

Exhibit L

Part 2 of 3

surface epitopes to probe biofilms for the location of particular organisms has the advantage of detecting a functional surface component in a complex biofilm. The disadvantage of this technique is the extensive characterization of the antibody required to determine its specificity. In some situations, even highly specific monoclonal antibodies react with an antigen, such as phosphorylcholine, which is found on several genera of oral bacteria (55). Such broad cross-reactivity may be useful for detecting cells expressing the antigen but does not permit identification of species in a mixed-species community. However, in other situations, it is likely that a functional surface receptor polysaccharide or cognate adhesin is found only on certain species and thus would map both a function and taxonomy. We have used both antibodies and green fluorescent protein to identify bacteria in dual-species biofilms *in vitro* (5, 120). A summary of this study follows.

When unamended sterile saliva is the nutrient source for monoculture biofilms, *S. gordonii* DL1 is capable of growth, whereas *S. oralis* 34 and *A. naeslundii* T14V are unable to grow (120). Each of these species coaggregates with the other two, and we tested the possibility that coaggregation between *S. gordonii* and either *S. oralis* or *A. naeslundii* in a biofilm might enhance communication with and growth of the latter two species. In pairwise combination, *S. gordonii* grew independently of the other two organisms; neither *A. naeslundii* nor *S. oralis* grew to any significant level. Rather, it seems that *S. oralis* and *A. naeslundii* were simply retained in coculture with *S. gordonii*. However, when *S. oralis* and *A. naeslundii*, the two species that failed to grow independently on saliva, were introduced sequentially into a flowcell, they coaggregated and grew luxuriantly (120). Moreover, the amount of growth exhibited in this mutualistic interaction was much greater than the independent growth by *S. gordonii*. These data provide a dramatic example of the consequences of mutualism and suggest that independent growth may not be the most advantageous strategy in complex communities.

Keeping in mind that both *A. naeslundii* and *S. oralis* are capable of binding to receptors in the salivary conditioning film, sequential addition of these two species to the flowcell could result in random and independent adherence to the saliva-coated surface. However, immediately after the unbound cells are removed by salivary flow, nearly all of the cells of the second species, *A. naeslundii*, were coadherent with the previously bound *S. oralis* cells (Fig. 4A). A preference for coadherence clearly occurs, because the two species are seen in juxtaposition rather than in a random distribution over the substratum. Examination of the coadherence in the Z dimension (Fig. 4, Z section below panel B) reveals that the red-stained cells (actinomycetes) are predominantly localized on top of the green-stained cells (streptococci), confirming the binding of the actinomycetes to the already adherent streptococci. After overnight saliva flow, extensive growth of both species is obvious (Fig. 4C and D). The biomass of both species increased laterally as well as axially, and the biofilm appeared denser (Fig. 4, Z section below panel D). Thus, retention through coadherence appears to lead to cooperative growth on saliva for these two species.

Even simple retention of a species without growth in a biofilm can be of critical *in vivo* significance. Retention is the principal requirement for growth in a flowing environment

such as the oral cavity. It allows communication signals synthesized after cell contact to enter the nascent environment and thereby encourage development of other, more favorable interbacterial relationships. For example, for the species discussed above (Fig. 4) (120), retention of *S. oralis* in an *S. gordonii*-dominated biofilm could enable *S. oralis* to wait for introduction of a more favorable partner, such as *A. naeslundii*, to join the biofilm community. Coadherence of *A. naeslundii* to *S. oralis* could then result in luxuriant growth of the partners. In addition, rapid growth of *S. oralis* and *A. naeslundii* could diminish growth of *S. gordonii* through competition for the same nutrient. Indeed, it is known that *S. oralis* is a dominant early colonizer of the tooth surface, while *S. gordonii* is present in lower numbers (114, 115). The ability to form advantageous partnerships and, by comparison, the ability to grow independently represent two of the physiological communication strategies that may occur in developing early dental plaque communities.

Another form of communication among oral bacteria in dental plaque is the exchange of free DNA from lysed cells to transformation-competent cells. Transformation of *S. gordonii* has been shown to occur *in vitro* with plasmid DNA in human saliva (104). The half-life for transforming activity of both plasmid and chromosomal DNA is only 5.7 s in saliva in the mouth, but 5.7 s is sufficient for DNA uptake and protection from salivary nucleases (105). Natural genetic transformation has been reported for many oral streptococci in planktonic cultures and occurs by induction of genetic competence through a competence-stimulating peptide (CSP) signaling system (61, 97). Competence in these bacteria is a quorum-sensing phenomenon, where the quorum size required may be species and strain specific. The products of at least six genes, *comAB*, *comX*, and *comCDE*, are involved in CSP signaling. CSP is encoded by *comC* and is usually found in a genetic locus with *comD* and *comE*, which encode a histidine kinase and a response regulator, respectively. A mutation in *comD* was identified in a screen of *S. gordonii* mutants with biofilm formation defects (94). Thus, it appears that oral streptococci in biofilms may communicate by a quorum-sensing CSP signaling system.

Cvitkovitch and coworkers discovered the quorum-sensing CSP signaling system in *Streptococcus mutans* and showed that this system is essential for genetic competence in *S. mutans* (89) and that it is involved in biofilm formation (91). Knockout mutants defective in *comC*, *comD*, *comE*, and *comX* were constructed and compared with the wild type. All of the mutants formed altered biofilms. Either exogenous addition of CSP or complementation of mutant *comC* with a plasmid containing wild-type *comC* restored the wild-type biofilm phenotype to the *comC* mutant (91). However, neither addition of CSP nor complementation showed any effect on the biofilms formed by the other *com* mutants. This quorum-sensing system also functions to regulate acid tolerance in *S. mutans* biofilms formed *in vitro* (90). Densely packed cells are more resistant to killing by low pH than are cells in less populated regions of the biofilm (90).

Accompanying the discovery of the CSP system is the evidence that biofilm growth of the streptococcus greatly enhances both competence induction and uptake and integration of a variety of plasmid and chromosomal donor DNAs (89).

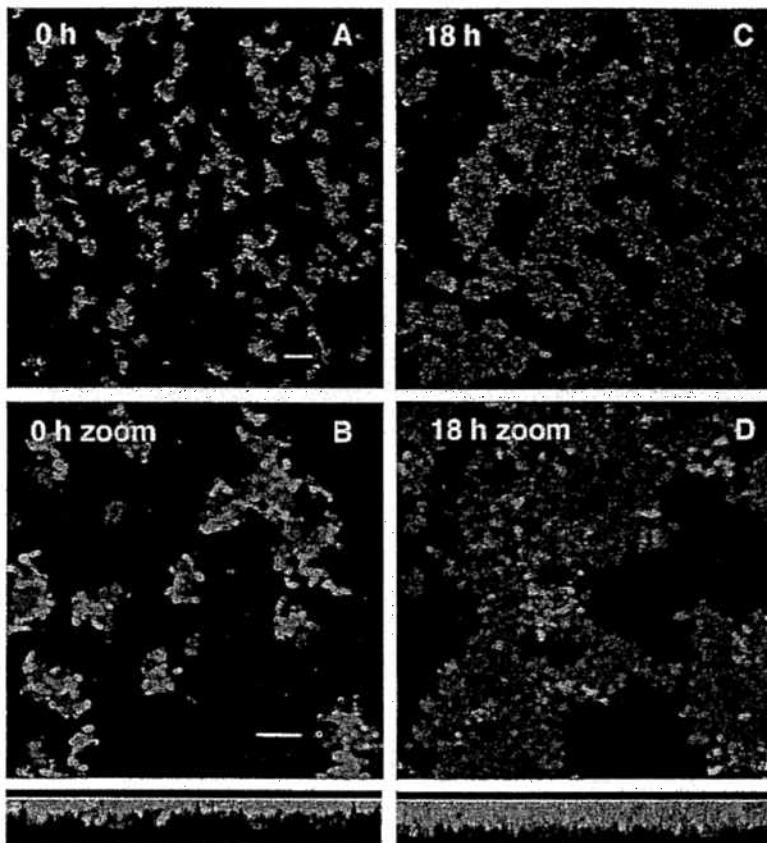


FIG. 4. Representative confocal scanning laser microscopy images of a two-species biofilm formed by *S. oralis* 34 and *A. naeslundii* T14V. Maximum projection images were taken at 0 h (A and B) and 18 h (C and D) of salivary flow. *S. oralis* 34 was incubated statically in a saliva-coated flowcell for 15 min before initiation of salivary flow at 0.2 ml/min for 15 min. *A. naeslundii* T14V was then added and incubated statically for 15 min, and salivary flow was resumed for 15 min (equals time zero). *S. oralis* 34 cells were labeled with rabbit anti-*S. oralis* 34 serum (gift of J. Cisar), followed by indocarbocyanine-conjugated goat anti-rabbit immunoglobulin antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.); indocarbocyanine fluorescence is presented in green. *A. naeslundii* T14V was labeled with a mouse monoclonal antibody against type 1 fimbriae (gift of J. Cisar), followed by indocarbocyanine-conjugated goat anti-mouse immunoglobulin antibody (Jackson ImmunoResearch Laboratories); indocarbocyanine fluorescence is presented in red. *A. naeslundii* cells are frequently located in direct proximity to *S. oralis* cells. After 18 h of saliva flow, growth of both genera is apparent. Dimensions of the regions displayed are 250 μ m by 250 μ m (x-y perspectives; A and C) and 83 μ m by 83 μ m (x-y perspectives; B and D; 3 \times zoom of the center portion of the upper panels). Rotation of the maximum projection to display the x-z perspective (83 μ m by 10 μ m) is shown below panels B and D. The substratum position in the x-z perspective is indicated by the white line. Bars, 20 μ m (A and C) and 10 μ m (B and D). Microscopic observations and image acquisition were performed on a TCS 4D system (Leica Lasertechnik GmbH, Heidelberg, Germany).

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With biofilm cells, transformation frequencies 10 to 600 times the rate seen with planktonic cells were observed and were maximal after 8 to 16 h of growth. Lysed cells in the biofilm could act as donors of chromosomal DNA, indicating that communities of living and dead cells may share information through genetic exchange. DNA could also act as a nutrient source for the growing biofilm (46), or possibly, extracellular DNA may contribute to initial establishment of oral bacterial biofilms in a manner similar to that recently shown for biofilms formed by a nonoral bacterium (153).

In another example, transfer of native conjugative transposon *Tn916*-like elements encoding tetracycline resistance (*Tet*^r) from one oral streptococcal species to another in a model biofilm was recently reported (126, 127). The significance of this finding is twofold: (i) tetracycline is used to treat

periodontal disease, and *Tet*^r elements circulating within dental plaque would thwart treatment as well as potentially transfer *Tet*^r to transient residents on their way to other body sites, and (ii) DNA transfer favors the possibility of genetic manipulation of these organisms and will thus facilitate molecular characterization of communication in biofilms. Thus, significant progress has been made in answering the question of whether DNA is transferred among bacteria in densely packed environments such as dental plaque.

Imaging by conventional confocal microscopy is difficult with thick specimens. Two-photon excitation microscopy is a potentially valuable tool for resolution of cell shapes at depths greater than 100 μ m. Two-photon excitation microscopy coupled with fluorescence lifetime imaging microscopy has been used to study pH gradients in biofilms formed by a consortium

of 10 species of oral bacteria (150). Pulses of 14 mM sucrose were supplied to the consortium to elicit acid production. Fluorescence decay of carboxyfluorescein was used to examine pH within the biofilm. Distinct microzonal variations in pH and sharp pH gradients were observed, indicating spatial heterogeneity of response to the same metabolic stimulus. This observation provides evidence for architectural organization of consortia and metabolism within the biofilm. Distinct microbial communities occur within the larger biofilm architecture and include a range of individual responses. Cells at the border of a niche are situated between cells that are responding by producing acid from sucrose and cells outside the niche responding in a different way. Thus, consortia can be understood to consist of physiologically heterogeneous species that respond differently to a given external stimulus. For example, the response may be growth, which may protect other members if the growing cells modify the local environment.

Metabolic Communication

The examples of metabolic communication discussed here are limited to interactions in which at least one organism benefits. This arena of metabolic communications among oral bacteria has been reviewed extensively (57, 73, 84, 100). Beneficial interactions may occur through the excretion of a metabolite by one organism that can be used as a nutrient by a different organism or through the breakdown of a substrate by the extracellular enzymatic activity of one organism that creates biologically available substrates for different organisms. An example of the latter enzymatic activity is sequential hydrolysis of a complex glycoprotein by several bacteria acting in sequence on the product of a previous bacterium's action, as has been shown for oral streptococci (18).

Within the oral cavity, bacteria form multispecies communities that are distinguishable primarily by their location (supragingival versus subgingival versus epithelial). The subgingival community has the highest species richness and the greatest capacity for pathogenic outcome, such as periodontal tissue destruction. In an examination of cocultures of putative periodontal pathogens, such as *P. gingivalis* and *T. denticola*, cocultures produced more biomass than was observed in the respective monocultures; most of the coculture biomass was in the form of cell aggregates, and the coculture was transferable over at least five successive inoculations (56).

Cell-free supernatants from monocultures were tested for the ability to stimulate growth of the companion organism. The supernatant from cultures of *P. gingivalis* could increase the growth of *T. denticola* and vice versa. Succinate was found in the *T. denticola* supernatant and was utilized by *P. gingivalis* monocultures, as determined by lowered concentrations of succinate during growth. The *P. gingivalis* supernatant contained six fatty acids, including isobutyric acid, and although none of those acids was removed by *T. denticola* monocultures, isobutyric acid was found to stimulate growth of *T. denticola* monocultures when added as a supplement at a concentration lower than that at which it occurred in the *P. gingivalis* supernatant (56). These results imply that cross-feeding between *P. gingivalis* and *T. denticola* occurs and that *T. denticola* requires only minor amounts of *P. gingivalis*-produced isobutyric acid for maximal growth stimulation.

About 60 oral species of *Treponema* have been identified (123), and spirochetes constitute a large percentage of the total oral bacterial numbers. Accordingly, a large *T. denticola* population could benefit greatly through interaction with a small *P. gingivalis* population. In a separate study, a stimulatory effect of *P. gingivalis* supernatant on *T. denticola* growth was attributed to proteinaceous substances (111); this study did not examine the inverse interaction. Thus, synergistic interactions between *P. gingivalis* and other anaerobic bacteria such as oral spirochetes yielded increased growth and may contribute to increased virulence of these potential periodontal pathogens.

To investigate the outcome of multispecies interactions on virulence, *P. gingivalis* and *F. nucleatum* were tested in a murine subcutaneous lesion model. Simultaneous injection of these bacteria at the same site resulted in larger lesions and higher morbidity than did injections of a single species (40). In a concurrent study, this synergistic effect was shown to occur even when each bacterial strain was injected separately on opposite sides of the same animal (45). Thus, this polymicrobial infection demonstrated cooperativity in virulence outcome.

Veillonellae are anaerobic gram-negative cocci that exist in supra- and subgingival plaque communities, where they make up 1 to 5% of the total cultivatable anaerobic bacteria (80). *Veillonellae* can utilize short-chain organic acids, especially lactate, for growth. These organic acids are excreted by streptococci during growth on sugars and are the basis for the metabolic communication documented in vitro (106) and in vivo in gnotobiotic rats (107, 149). Also, it has been shown in vivo that *veillonellae* are not capable of colonizing the tooth surface without streptococci as metabolic partners and that larger populations of *veillonellae* develop in coculture with streptococci that recognize them as a coaggregation partner than in coculture with streptococci with which they do not coaggregate (101).

The conversion of the lactic acid formed by streptococci to less potent acids, such as acetic acid, by *veillonellae* has been assumed to reduce caries susceptibility in the host, although little experimental evidence supports this hypothesis. Instead, a molecular study suggests that *veillonellae* are present together with streptococci in carious lesions (8).

While this molecular approach does not establish causality, it does indicate the types of bacteria present at specific sites in health and in disease. Such studies have identified reproducible bacterial communities found at particular disease sites (137), and thus, they identified bacterial populations that may arise through interaction with one another. Studies of biofilm architecture at these sites together with studies of the physiological consequences of that architecture will be required to sort out the occurrence and nature of metabolic communication.

In Vivo

Significant advances have been made in clinical research directed at understanding the architecture of supragingival and subgingival dental plaque *in situ*. From the five reports discussed here, a picture of dental plaque architecture is emerging.

Supragingival plaque is formed on the outwardly visible

enamel surface of teeth. It has been studied *in situ* by bonding to teeth a device consisting of an enamel piece with an adherent nylon ring (157). After removal from the tooth surface, the supragingival plaque biofilm formed within the nylon ring on the enamel substratum is examined by confocal scanning laser microscopy. The voids observed among the adherent biomass (157) appear to be filled with fluid, as has been reported for several *in vitro* biofilms composed of one or only a few species (32, 86, 159). On the basis of these observations, voids may serve as avenues for metabolic communication as well as for signaling molecules and antimicrobial compounds.

One method used to study the diffusion of molecules and the efficacy of antimicrobials in undisturbed supragingival plaque was to measure penetration of differential stains into plaque formed *in vivo*. In these studies, a bovine dentin disk was bonded to an acrylic appliance that was worn by a human subject (159). The disk contained three parallel 200- μm -wide grooves, which were about 500 μm deep and served as the location for plaque accumulation. Upon removal of the appliance, the disks were broken in half along the middle groove. One half was covered with 0.2% chlorhexidine for 1 min; the other half was the control. After treatment, both halves were rinsed with saline, stained with a mixture of ethidium bromide and fluorescein diacetate, and viewed by confocal scanning laser microscopy. Ethidium bromide, a red fluorescent nucleic acid stain, permeates only cells with damaged cell membranes, and fluorescein diacetate penetrates all cells but is nonfluorescent until the green fluorescein moiety is freed by intracellular esterases.

The biofilms grew up to 65 μm thick, and the plaque structure appeared diffuse, with morphologically heterogeneous cell shapes. Both stains penetrated the entire thickness, indicating that there was no barrier to small molecules in these plaque biofilms. In contrast, as measured by cell death (ethidium fluorescence), the antimicrobial chlorhexidine had an effect primarily near the lumen (159). Only the cells near the lumen may have been killed because (i) cells located deeper are more resistant to killing, (ii) antimicrobial action is titrated by contact with surface-exposed biomass and thus the dose of antimicrobial received deeper in the biofilm is ineffectual, or (iii) biofilms may not be indiscriminately open to all molecules throughout their thickness. Furthermore, as plaque biofilm community composition changes, the gating and selectivity of diffusible molecules may also change in response to communication signals. Biofilms may select and gate putative communication signals, and the biofilm inhabitants may temporally regulate such gating. Additional studies to determine the selective porosity of biofilms to signaling molecules will help answer this question.

To study subgingival plaque, oral biofilms formed below the gum line, researchers either use a model system or extract teeth and examine the periodontal region directly. One model system involves three different materials placed into periodontal pockets of patients with rapidly progressing clinical periodontitis (151). A membrane of polytetrafluoroethylene (a material used to cover superficial defects after surgery that can be colonized by plaque bacteria), gold foil (for scanning electron microscopy), and dentin were used. Periodontal pocket depths were approximately 8 mm, and the materials were held in place for 3 to 6 days, yielding biofilms 40 to 45 μm thick.

Analyses were conducted by confocal scanning laser microscopy with fluorescently labeled 16S rRNA-directed oligonucleotide probes and by electron microscopy. Probes specific for *Treponema* spp. and general bacterial (EUB338 [1]) probes were used to detail the architecture of the periodontal biofilm *in situ*. The deepest zones of the pockets were colonized principally by spirochetes and gram-negative bacteria, whereas shallow regions contained mostly gram-positive cocci (151). The composition of the carrier material had little influence on colonization. Complex communities of interspersed, morphologically distinct cell types were commonly observed, as were characteristic organized arrangements of mixed cell types, such as rosettes consisting of a gram-positive central bacterium surrounded by gram-negative rods.

The rosettes and other mixed-cell-type arrangements seen in situ bear striking similarity to those seen *in vitro* during coaggregation studies (74) and in other *in situ* electron microscopy studies (69, 93). Species-specific FISH probes allowed this first unequivocal demonstration of the spatial arrangement of spirochetes *in situ* within the periodontal biofilm (151). As additional species-specific oligonucleotide probes are designed, the entire architecture of dental plaque *in situ* can be elucidated.

In another study examining spatial relationships, Noiri et al. (112) extracted teeth from sites with periodontal pocket depths of approximately 8 mm from patients with advanced adult periodontitis. Intact periodontal pockets were preserved, and the localization of five species, *Prevotella nigrescens*, *F. nucleatum*, *T. denticola*, *Eikenella corrodens*, and *Actinobacillus actinomycetemcomitans*, was investigated by immunohistochemistry. Localization of bacteria within the periodontal pocket was analyzed by separating the pocket into nine zones. Vertical zones extended from the gingival margin to the deep pocket and were categorized as shallow, middle, and deep. Horizontal zones, from the tooth surface to the pocket epithelial surface, were called tooth attached, unattached, and epithelium associated.

The results of the immunohistochemistry revealed that *P. nigrescens* is located at the epithelium-associated plaque area in the middle pocket zone. The middle and deep pocket zones of the unattached area were preferentially colonized by *F. nucleatum* and *T. denticola*, but these two bacteria could be found in all zones except one or two of the shallow zones. *E. corrodens* was located primarily in tooth-attached plaque zones, whereas *A. actinomycetemcomitans* was found infrequently and only in the middle, unattached plaque zone. *F. nucleatum* was found in samples from 9 of 15 patients, whereas the other species were found in six or fewer samples. In an earlier study, this research group showed that an antiserum reactive against actinomycetes detected cells predominantly in the tooth-attached, shallow and middle zones (113).

Thus, it appears that each oral bacterial species colonizes preferred sites within the subgingival plaque architecture. This selective colonization suggests that species organize into communities through communication with their host and with other species that occupy the host's substrata.

SOLUBLE-SIGNAL COMMUNICATION AMONG ORAL BACTERIA

Transfer of genes by competence-inducing pathways is one of the most-studied forms of communication by oral bacteria.

This method of communication is mediated by competence-stimulating peptides (CSPs) (62), which are small, cationic peptides of 14 to 23 amino acid residues that are produced by at least 10 species of oral streptococci (62, 89). Since several reviews and book chapters have discussed the important role of CSPs (62, 97, 108), this method of oral bacterial communication will not be discussed in detail here. Likewise, readers are referred to other reports and reviews describing the roles of sex pheromones and their inhibitor peptides in the well-described mating system of *Enterococcus faecalis* (3, 29, 30).

Signaling by soluble molecules also occurs between *Streptococcus salivarius* and *Streptococcus pyogenes* by the lantibiotic peptides that modulate their own and interspecies lantibiotic synthesis and that may be a mechanism for controlling susceptible streptococci in the human oral cavity (148). Many oral bacteria produce bacteriocin peptides; some are lantibiotic peptides, and others are nonlantibiotic peptides (65, 125). These peptides are thought to influence the ecology of these bacteria, but their mechanism of communicating this influence is unknown. One investigation was conducted with several oral streptococci known to coaggregate with each other (147). One strain, *S. gordonii* DL1 (Challis), produced a bacteriocin, and five strains were sensitive to the bacteriocin, but neither the production of nor sensitivity to the bacteriocin could be correlated to the ability to coaggregate, suggesting that cell-cell attachment and bacteriocin production are not related.

Physical communication is ubiquitous among oral bacteria in that all of the human oral bacteria tested coaggregate with at least one other genetically distinct species (73). Recently, several examples of production of a quorum-sensing molecule called autoinducer-2 (AI-2) have been reported in oral bacteria (15, 26, 48, 49). We will propose some ideas on possible methods of communication involving AI-2 in a mixed-species community.

Autoinducer-2

Methods of communication among genetically identical cells are likely to be different from the communication signals exchanged among species. AI-2 is proposed to be a signal mediating messages among different species in a mixed-species community (108, 133), distinguishing it from the family of acyl homoserine lactones, typified by autoinducer-1, that regulate gene expression in genetically identical cells (6, 50). No evidence of acyl homoserine lactone production by oral bacteria has been found, suggesting that oral bacterial species do not use acyl homoserine lactones for signaling (49, 154). In contrast, *luxS*, the gene encoding the enzyme essential for production of AI-2, is present in several genera of oral bacteria (15, 26, 48, 49). AI-2 was discovered in the marine bacterium *Vibrio harveyi*, in which it is a signal molecule that regulates bioluminescence (7). A set of bioluminescent reporter strains of *V. harveyi* were constructed (139, 140) and have been used by many investigators to assay for the production of AI-2 by other bacteria. Through these studies, AI-2 has been implicated in gene regulation in several species besides *V. harveyi*, including *Streptococcus pyogenes* (98), *Escherichia coli* (35), *Salmonella enterica* serovar Typhimurium (141), and two human oral bacteria, *A. actinomycetemcomitans* (48) and *P. gingivalis* (15, 26).

Signaling by AI-2 to produce light in *V. harveyi* is accom-

plished through binding of AI-2 to LuxP, followed by a phosphorylation cascade (25). The structure of AI-2 bound to its primary receptor LuxP in *V. harveyi* was recently determined to be a furanoyl borate diester (25). LuxP is an AI-2 sensor protein and is located in the periplasmic space of *V. harveyi*. AI-2 is produced from *S*-adenosylmethionine through several steps, including the required enzymatic conversion of the intermediate *S*-ribosylhomocysteine by LuxS to 4,5-dihydroxy-2,3-pentanedione, which is unstable and is predicted to cyclize spontaneously (133, 134) into a variety of molecules called pro-AI-2 before forming a mature AI-2-LuxP complex (25). The site of borate addition to form AI-2 is unknown and may be in the AI-2-producing cell, outside the cell, or in the recipient cell (25). AI-2 and conserved *luxS* have been found in more than 30 bacterial species (108, 140), including both gram-positive and gram-negative organisms. Considering the broad representation of *luxS* among bacteria, AI-2 has been proposed to be a universal interspecies signal (108, 133), but this role has yet to be demonstrated in an ecologically relevant consortium of bacteria.

Alignment of LuxS sequences from 26 organisms revealed 23 invariant amino acid residues (64). An alignment of the amino acid sequences deduced from the *luxS* genes of four oral bacteria, *A. actinomycetemcomitans*, *P. gingivalis*, *S. gordonii*, and *Streptococcus mutans*, as well as *V. harveyi* shows that all 23 invariant amino acids are present (Fig. 5). The streptococcal LuxS sequences are 83% identical to each other and 30 to 40% identical to the other three sequences, demonstrating that the gram-positive streptococcal enzymes are more closely related to each other than to enzymes from the three gram-negative species in this alignment. The *A. actinomycetemcomitans* LuxS sequence is 72% identical to *V. harveyi* LuxS but only 31% identical to the LuxS sequence of *P. gingivalis*, which is 29% identical to *V. harveyi* LuxS. Finding *luxS* in both gram-positive and gram-negative strains of oral bacteria suggests a role for AI-2 in mixed-species communities such as dental plaque.

In a survey of 16 species of oral bacteria, *F. nucleatum*, *P. gingivalis*, and *Prevotella intermedia* produced high levels of AI-2 under the conditions tested (49). Another periodontal bacterium, *A. actinomycetemcomitans*, was subsequently reported to produce AI-2 (48). These AI-2-producing species are all involved in the polymicrobial infection known as periodontal disease. Additional oral species are being surveyed for production of AI-2 with the expectation that AI-2-mediated communication may play a central role in gene regulation in mixed-species communities.

Two oral species, *P. gingivalis* and *A. actinomycetemcomitans*, were examined for regulation of gene expression by AI-2 (15, 26, 48). Based on the *V. harveyi* reporter assay, conditioned medium from these two oral bacteria elicited a response that was approximately 10 and 20%, respectively, of that of the *V. harveyi* positive control (26, 48). As measured by reverse transcription-PCR, two genes relevant to hemin acquisition were reported to be induced in a *P. gingivalis luxS* mutant, and three other genes were repressed, indicating a response to *luxS* inactivation (26). Expression levels of genes that were altered in the *P. gingivalis* mutant were also affected by exposure of the mutant to conditioned medium from a recombinant *E. coli* strain harboring the *A. actinomycetemcomitans luxS* gene on a plasmid (48). Additionally, as measured by reverse transcrip-

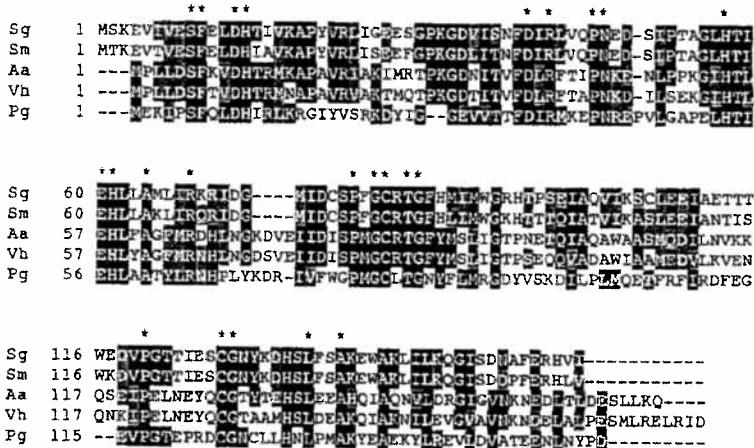


FIG. 5. Alignment of the deduced amino acid sequences of *luxS* from *S. gordonii* DL1 (Sg; accession number AY081773), *S. mutans* UA159 (Sm; www.genome.ou.edu/smutsans.html), *A. actinomycetemcomitans* HK1651 (Aa; www.genome.ou.edu/act.html), *V. harveyi* BB120 (Vh; accession no. AF120098), and *P. gingivalis* W83 (Pg; www.tigr.org). Consensus of at least 50% identical amino acid residues is denoted by black boxes. Asterisks are placed above the 23 amino acid residues that were shown to be invariant in 26 LuxS sequences aligned by Hilgers and Ludwig (64).

tion-PCR, exposure of *A. actinomycetemcomitans* to conditioned medium from the recombinant AI-2-producing *E. coli* strain described above resulted in increased transcription of *afuA*, which encodes a periplasmic protein involved in iron transport (48). As measured by a cytotoxicity assay, leukotoxin production was enhanced in *A. actinomycetemcomitans* cultures exposed to conditioned medium from the parent strain or from the recombinant *E. coli* strain described above (48).

In a separate study (15), it was reported that a *P. gingivalis* *luxS* mutant produced approximately 30% less Lys-gingipain and about 45% less Arg-gingipain, two cysteine proteases that are synthesized at high cell densities. While the regulation of genes involved in iron acquisition, protease synthesis, and leukotoxin production appears to be influenced by the presence of AI-2 in *P. gingivalis* and *A. actinomycetemcomitans*, it is not yet clear whether the differential regulation of these genes affects the virulence of these oral pathogens *in vivo*. Nonetheless, the pairing of *P. gingivalis* and *A. actinomycetemcomitans* has provided a starting point for demonstrating that AI-2 may serve as an interspecies signal (108, 133) in two organisms that coexist in the same ecological niche.

Modeling Oral Bacterial Mixed-Species Communication by AI-2

A LuxP homologue was found in *A. actinomycetemcomitans* (48) but not in *P. gingivalis* (26). This leaves open the possibility that oral bacteria may have alternate binding proteins for AI-2 that may be like the periplasmic ribose-binding proteins of *E. coli* and *S. enterica* serovar Typhimurium (7) and a recently discovered transporter of AI-2 in *S. enterica* serovar Typhimurium (141), which are all homologous to LuxP of *V. harveyi*. In accordance with the concept of alternative binding proteins, it is possible that a variety of cognate fits of pro-AI-2 molecules and binding proteins may occur within a mixed-species community. The binding affinity of each pro-AI-2/binding protein

pair may be characteristic of a species and thus permit numerous potential cognate fits within mixed-species communities. Thus, by using their respective cognate binding proteins, many species could simultaneously sample a mixture of pro-AI-2 in their microenvironment. Likewise, a mixture of pro-AI-2 in the microenvironment may be sampled differently by different bacteria because the concentration of pro-AI-2 molecules is critical for specificity in regulating gene expression. Thus, the capacity of AI-2 to serve as a universal signal within a mixed-species bacterial community may stem from individual organisms' abilities to bind and respond to unique molecules within the environmental pool of pro-AI-2.

It is challenging to postulate attractive mechanisms of mixed-species communication by the proposed universal interspecies signal AI-2. First, some but not necessarily all of the species in the community must express an active LuxS, since it is required for synthesizing pro-AI-2. Second, to be universal, all the species must respond to pro-AI-2. Third, as culture supernatants from a variety of genera elicit light production in the appropriate *V. harveyi* reporter strains, at least some of the extracellular pro-AI-2 molecules produced by different species must have identical structures. However, not all of the pro-AI-2 molecules need to be identical. Thus, a mixture of pro-AI-2 molecules may be a significant factor in the ability of an organism to compete by sensing, selecting, and responding to a particular isomer within the blend of pro-AI-2 molecules. Analogs of AI-2 such as 4-hydroxy-5-methyl-furanone were found to stimulate the *Vibrio* reporter strain to produce light when added at 1,000-fold the concentration of AI-2 required for light production (134). This demonstrates that, although less efficient, the LuxP of *V. harveyi* will bind and respond to molecules other than naturally synthesized pro-AI-2. Assuming that several and perhaps all of the species in a mixed-species community such as dental plaque are producing pro-AI-2, the species-specific mode of communication may be based simply on